Exhibit 1

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An immunotherapeutic treatment against flea allergy dermatitis in cats by co-immunization of DNA and protein vaccines☆

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ABSTRACT

Flea allergy dermatitis (FAD) is considered a harmful and persistent allergic disease in cats, dogs and humans. Effective and safe antigen-specific treatments are lacking. Previously we reported that the simultaneous co-immunization with a DNA vaccine and its cognate coded protein antigen could induce antigen-specific iTreg cells (inducible Treg cells); demonstrating its potential to protect animals from FAD in a murine model. Its clinical efficacy however, remains to be demonstrated. In this report, we clinically tested this protocol to treat established FAD in cats following flea infestations. We present data showing a profound therapeutic improvement of dermatitis in these FAD cats following two co-immunizations, not only in relieving clinical symptoms, but also the amelioration of the allergic responses, including antigen-induced wheal formation, elevated T cell proliferation, infiltration of lymphocytes and migration of mast cells to the sites. This study demonstrates that a co-immunization approach as described can be used to treat flea-induced allergic disease in animals, thus implicating its potential for a practical clinical application.

1. Introduction

Allergic reactions to insect stings are a common and often serious medical problem, both in humans and animals [1–4]. FAD is one of the most severe skin allergies caused by flea infestations in dogs, cats, and even in humans [5,6]. Further, flea bites can cause scratch-related secondary infections as a consequence of the inflammatory irritation of host's erythema, papules, crusts, and alopecia [7,8]. Previous work found that the pets rarely became desensitized to the bites of flea once they have been made allergic to them [9,10]. So besides the elimination of the flea, the alleviation of the animal's distress becomes the challenging problem. Current therapies for this disease include desensitization therapy or using some types of pharmacological intervention [11]. However, each of these therapeutic approaches has some disadvantages [12]. For example, anti-histamine medications could increase drowsiness, dry mouth, difficulty in urination, and constipation; whereas, desensitization therapy may cause life-threatening anaphylactic shock. Other disadvantages of these therapies are the high possibility of recrudescence and requirement for long-term treatments [13]. Therefore, novel, effective therapeutic approaches are needed and should be developed in order to overcome unwanted side-effects. It has been proposed that DNA vaccine encoding allergen could down-regulate the allergic response by redirecting immunity from a Th2 type to Th1 type. Th1 cells induced by DNA vaccine could protect against allergic disease by inhibiting the proliferation and development of Th2 cells [14] and IgE production [15]. However, other reports demonstrated that antigen-specific Th1 cells were ineffective in reducing the airway hyper-reactivity induced by Th2 cells and caused serious airway inflammation [16]. Thus, immunotherapeutic approaches using Th1 cell responses against allergic diseases appear to be rather more complicated.

T regulatory cells, including the naturally occurring thymus derived $CD4^+CD25^+$ Treg cells could be used to down-regulate immune responses [17,18]. Adaptive Tr1, mucosal induced Th3 cells and antigen-induced $CD4^+CD25^-$ Treg cells have been proposed to be used as immuno-regulators of auto-reactive

Abbreviations: FAD, flea allergy dermatitis; AIH, allergen induced immediate hypersensitivity; FSA1, flea saliva antigen 1; Treg cell, T regulatory cell; nTreg, nature occurred Treg; iTreg, inducible Treg.

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pathogenesis [19–21]. Various approaches have been explored to induce T regulatory cells to constrain the auto-reactive T cells [22,23]. In particular, induction of antigen-specific T regulatory cells targeted to allergy, asthma and autoimmune disease antigens is considered a promising immunomodulatory strategy [24]. Recently we have demonstrated in a mouse model, that T cell suppression can be induced by co-immunization with protein antigen and plasmid DNA coding the same antigen [25,26]. This suppression is apparently associated with the generation of antigen-specific $CD4^+CD25^-FoxP3^+$ iTreg cells secreting IL-10 and TGF-β cytokines. It would be very interesting to know if this induction of iTreg cells observed in the murine model could also be reproducible in a clinical setting, in other larger animals.

In this report, we extend our previous co-immunization study and demonstrate the effect of co-immunized DNA vaccine encoding flea saliva antigen 1 (FSA1) with FSA1 protein after inducing flea allergy dermatitis (FAD) by flea infestations in domestic cats. The clinical onset of FAD was significantly reduced and its inflammatory symptoms were also improved by the co-immunization protocol. We found that the improved therapeutic effects on FAD in cats were due to the suppression of inflammatory T cell reactions. Collectively, this result is the first such demonstration of using a co-immunization strategy that may be applicable in a clinical disease setting.

2. Materials and methods

2.1. Vaccine preparations

The DNA sequence from the full-length of flea salivary antigen (FSA1, GeneBank access no. AF102502) was synthesized and cloned into pVAX1 vector (Invitrogen Inc., USA). Recombinant FSA1 protein was cloned into pET28a and expressed in *E. coli* system. The pVAX-FSA1 expression was identified by RT-PCR analysis from the total RNA of transfected BHK21 cells after 72 h. The FSA1 protein was purified from pET28a-FSA1 transformed *E. coli* BL21 (DE3) according to a previous protocol [27]. The quantity and quality of the FSA1 protein were analyzed by Bradford assay, SDS-PAGE and Western blot, respectively. Plasmids and recombinant proteins are dissolved in saline at 1 mg/ml and stored at 4 °C before use.

2.2. Mice and immunization

Female C57BL/6 mice at 8–10 weeks old were purchased from Animal Institute of Chinese Medical Academy (Beijing, China) and received pathogen-free water and food. Mice were immunized with plasmid DNA at 100 μg, protein at 100 μg, or a combination of both at 100 μg each as the vaccine regimens, respectively, into tibialis anterior muscle on days 0 and 14.

2.3. T cell proliferation of mice

Single lymphocyte suspensions were obtained from spleens of each group, 7 days after the second immunization. T cell proliferation was performed by the MTT method after the FSA1 stimulation *in vitro* for 48 h. Following stimulation, cell proliferation was assessed by a colorimetric reaction after the addition of 20 μl of a 5 mg/ml MTT-PMS (Promega, USA) solution for 4 h; then the samples were centrifuged at 500 × *g* for 5 min and then adding 100 μl DMSO (AMRESCO, USA). Its colorimetric density was determined at 595 nm using a 96-well plate reader (Magellan, Tecan Austria GmbH).

2.4. Flow cytometric (FACS) analysis

T cells were isolated and stained with phycoerythrin (PE), fluoroscein isothiocyanate (FITC) or APC-conjugated mAbs in the presence of PBS for 30 min at 4 °C. For intracellular staining, T cells were stimulated with FSA1 protein (10 μg/ml) for 8 h and subsequently treated with monensin (100 μg/μl) for 2 h *in vitro*. The cells were blocked with Fc-Block (BD Phamingen, San Diego, USA) in PBS for 30 min at 4 °C before fixed with 4% paraformaldehyde and permeabilized with saponin. The cells were intracellularly stained with the appropriate concentrations of antibodies including PE- labeled anti-FoxP3- or PECy5- labeled anti-CD25 antibody, FITC-labeled anti-CD4 antibody or APC-labeled anti-IL-10 antibody 30 min at 4 °C, respectively. The cells were washed and analyzed with a FACScalibur using the Cell QuestPro Software (BD Bioscience).

2.5. Isolation of $CD4^+CD25^-$/$CD4^+CD25^+$ T cells and adoptive transfer

Single splenocyte suspensions were prepared from mice spleens and $CD4^+CD25^-$/$CD4^+CD25^+$ T cells were isolated and purified by using the MagCellect Mouse $CD4^+CD25^+$ T Cell Isolation Kit according to the manufacturer's protocol (R&D Systems, Inc., USA). The purity of the selected cell populations was 96–98%. The purified cells (1×10^6 per mouse) were adoptively transferred intravenously into C57/BL6 mice.

2.6. Induction of allergy response and intradermal test (IDT) in mice

At 24 h after the adoptively transferred, the mice were injected 100 μg/mouse flea saliva extracts (Greer Laboratories, Inc., Lenoir, NC, USA) and mixed with 1% aluminum hydroxide intraperitoneally. On days 7 after induction, the mice were injected flea extract intradermally with 1 μg/μl of flea extract on a non-lesional lateral thorax skin; PBS is used as a negative control and histamine is used as a positive control. The diameter of the skin reaction was measured 20 min after the challenge by using an engineering caliper. The result was calculated as an average of the three measurements.

2.7. Cats and flea

Pathogen-free domestic cats, 1–2 years old, were purchased from North China Pharmaceutical Group (Shijiazhuang, Hebei, China) and housed in animal facility at the Center for Disease Control and Prevention of China (Chinese CDC, Beijing, China) in the course of experiment. All experiments on cats were designed and operated according to international guiding principles for biomedical research involving animals. The sterile fleas were supplied by Chinese CDC.

2.8. Induction of FAD model

Ten cats were divided into FAD model group with six cats and four/group for naïve animals. Cats were separated on day 0 and infestated with 100 unfed fleas to induce the FAD model. The infestated cats were fed with the nitenpyram (Capstar, Novartis Animal Health, Greensboro, NC) [28] to remove the flea from their bodies on the 3rd day after each infestation. Naïve cats were also fed nitenpyram as negative control. This challenge cycle was repeated every other week for 4 times. The lesion assessments included: erythema, papules, crusts, scale, alopecia, and excoriation. Each lesion was scored by a blind observer using a scale from 0 to 3: with 0, no sign; 1, mild; 2, moderate; 3, severe. Three body sites were assessed; (1) back, from the head to the tail; (2) double lateral abdomen, from

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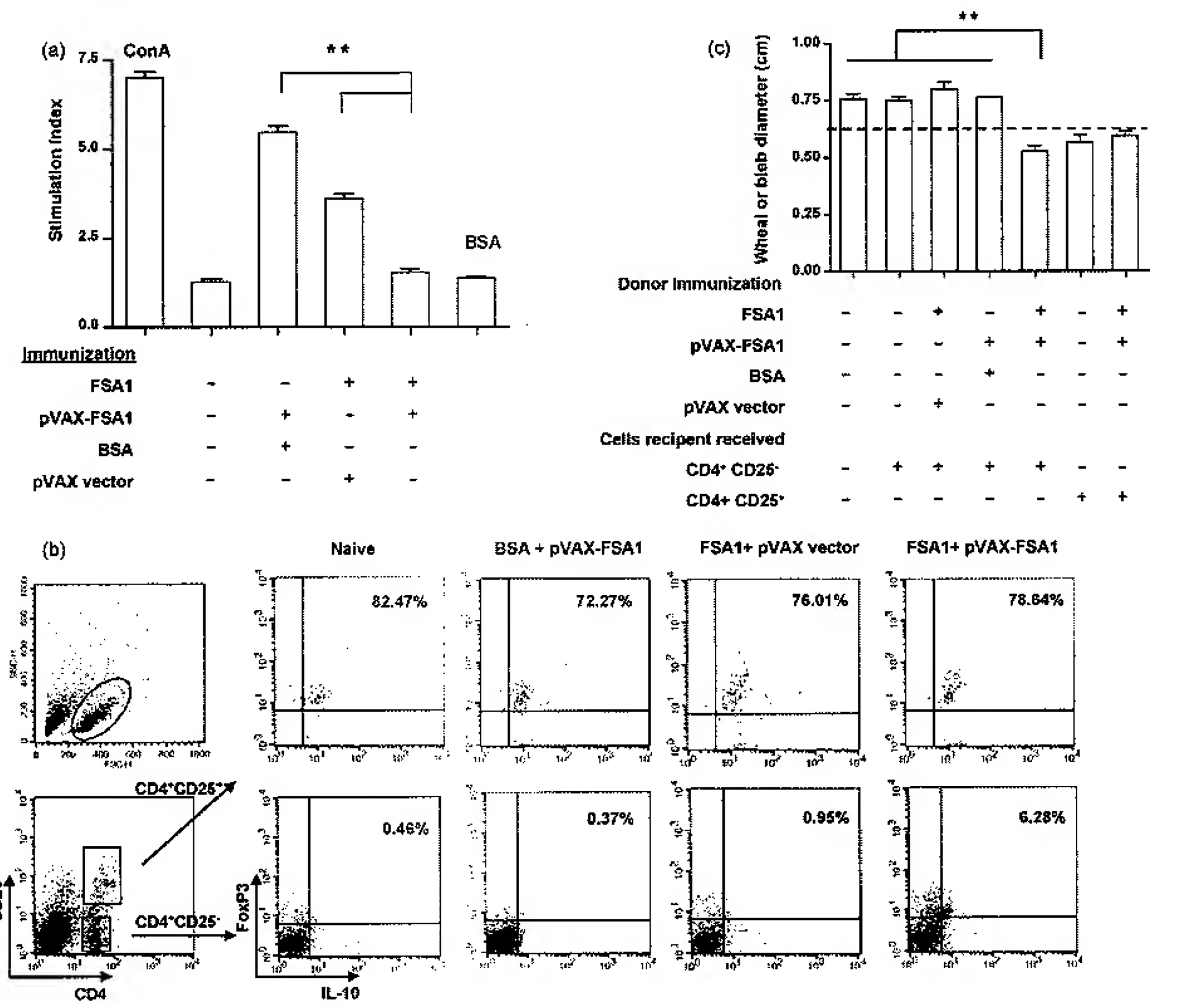

Fig. 1. Co-immunization suppresses allergic response to allergen. (a) Proliferative responses of T cells stimulated by the flea extract *in vitro* were performed on days 7 after the second co-immunization. Proliferation assay was analyzed by MTT method. Total T cells of mice on days 7 after the second co-immunization were analyzed for their intracellular cytokine productions of IL-10 and FoxP3 by FACS. (b) Both $CD4^+CD25^-$ (R5) and $CD4^+CD25^+$ (R6) T cells were gated for the co-expression of IL-10 and FoxP3. Results shown are representative of three independent experiments. Percentages represent percent of double positive cells. (c) The skin reaction (IDT) challenged with flea extracts was measured in the recipient mice 24 h later after receiving either $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells from syngeneic mice previously co-immunized. Results are representative of at least three independent experiments. The dashed line was indicated as the average reaction from the histamine and saline challenge. $^{**}P < 0.01$ compared with pVAX-FSA1 + BSA and FSA1 + pVAX vector mismatched control groups as indicated. ($n = 6$ mice per group).

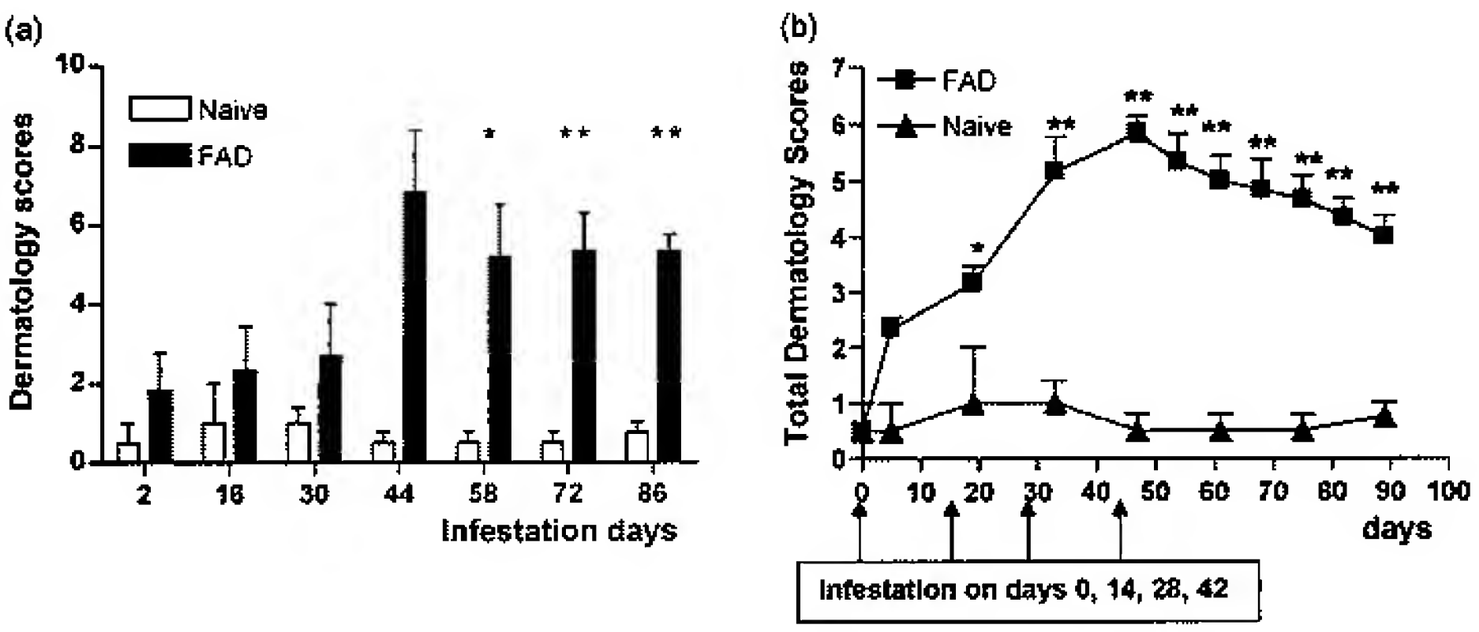

Fig. 2. The development of clinical signs of FAD model in cats. (a) The cats were induced into FAD with seven rounds of flea infestations, and then total sum of the dermatology scores included all types of lesions for all 3 body sites was counted. The cats without any infestation were observed as naive. (b) Duration of clinical onsets in FAD model in cats was detected. The total dermatologic scores including 4 cycles of infestations over 6 weeks of observation were plotted with 6 cats and the 4 cats were used for the negative control. $^{**}P < 0.01$, compared with naive group as indicated.

the scapula to the haunch; (3) flea triangle area, from laryngeal to the hypogastria.

2.9. Cat immunization

18 cats were induced for the FAD model by infestation with fleas for four cycles, and then separated into three groups with six animals each. The co-immunized group received 500 μg FSA1 protein + 500 μg pVAX-FSA1. The vector control group received 500 μg FSA1 protein + 500 μg pVAX vector. The non-relevant protein group received 500 μg pVAX-FSA1 + 500 μg BSA. All vaccines were formulated in PBS and used to immunize the cats intramuscularly on days 47 and 61.

2.10. Intradermal test (IDT) in cats

Intradermal tests (IDTs) were done following the protocol (Hillier and Deboer, 2001). On days 7 after the last challenge or days 7 after the last immunization, the cats were injected with 100 μl PBS containing 100 μg of flea extract on the lateral thorax skin of the cats intradermally; histamine was used as positive control, BSA used as an irrelevant stimulator, and saline used as the negative control. The size of reactive bleb on the skin was marked with a marker pen and measured perpendicularly and horizontally within 20 min after the challenge by a micrometer. The result was calculated as an average of the three measurements.

2.11. T cell isolation from peripheral blood monocytes of cats

PBMC was separated from peripheral blood. Blood was collected into a 50 ml conical tube, then adding 15 ml of HANK solution and mixing together. The samples were transferred into Lymphocyte separation solution, and then centrifuged at 500 × *g* at room temperature for 20 min. The cells in the middle opaque layer were PBMC, and then the T cells were purified by nylon wool and counted.

2.12. Determination of flea-specific antibodies

Sera were collected for flea antigen-specific antibodies testing by ELISA. The 96-well plates were coated with flea antigen at 4 °C overnight. After washing with PBST, the serum was added and incubated for 1 h at 37 °C, and then was treated with specific horseradish peroxidase-conjugated rabbit anti-cat IgE antibodies (SouthernBiotech, Birmingham, USA) for 1 h at 37 °C. The absorbance (450 nm) was measured using an ELISA plate reader (Magellan, Tecan Austria GmbH).

2.13. RT-PCR to test cytokines

Total RNA was isolated from T cells of cats, 7 days after the second immunization using TRIzol reagent (Promega). cDNA was synthesized and PCR was performed with each of the following primers: GAPDH, IL-4, IFN-γ, IL-5, IL-10. RT-PCR was performed with each of the following primers: GAPDH, IL-4, IFN-γ, IL-5 and IL-10 [29] according to the manufacturer's instructions (TaKaRa RNA PCR Kit). The PCR products were run in gels and stained with ethidium bromide.

2.14. Histology analysis

On days 7 following the second immunization, the skin biopsies of cats pretreated anesthetic were performed using a 3-mm punch on IDT site or lesion site from every group, and then the cats were dressing the antibiotics on wounds. The skin biopsies were fixed in 4% paraformaldehyde (Sigma, USA), and embedded in paraffin blocks. Sections were cut and fixed. Antigen retrieval was accomplished by boiling the slides in 0.01 M citrate buffer (pH 6.0) and followed by staining with hematoxylin (Sigma, USA) and eosin (Sigma, USA) or toluidine blue (Sigma, USA) for mast cells and analyzed under a light microscope for determining histological changes.

2.15. Statistical analysis

Statistical analyses were performed using the Student's *t*-test. if the value of $P < 0.05$, the data indicated significant differences.

3. Results

3.1. Co-immunization suppresses immune response to allergen in mice

As previously demonstrated, the epitopic sequences of the major allergen of flea saliva antigen 1 (FSA1) used in the co-immunization protocol protected mice from the allergic reaction [25,26]. To enhance the suppressive response, we sought to utilize the full-length cDNA of FSA1 which could provide a superior level of potency. Thus, the cloned FSA1 in both eukaryotic and prokaryotic expressing constructs were constructed. To examine their ability to induce iTreg cells, the T cell proliferation of mice immunized with different expressed regimens on days 7 after the second immunization was assessed. As shown in Fig. 1a, the FSA1 specific T cell proliferation was inhibited only in the mice co-immunized with the pVAX-FSA1 and FSA1 protein compared with other groups, which was consistent with the inhibition results we observed by using the epitopic DNA vaccine and peptide co-immunization protocol.

In order to eliminate the influence of unrelated vector and protein on the response, we co-immunized mice with pVAX-FSA1 plus BSA, or FSA1 protein plus pVAX vector, as the mismatched regimen controls, and these combinations exhibited no T cell impairment, but rather induced high levels of T cell proliferation (Fig. 1a).

The results indicate that the co-immunization of DNA vaccine, pVAX-FSA1, encoding the antigen matched with its cognate protein antigen, FSA1, resulted in an inhibition of antigen-specific T cells proliferative response, while the antigens in the mismatched combinations did not.

To confirm that the impaired T cell response is due to induction of iTreg cells as we previously reported in our epitopic co-immunization study [25,26], we analyzed T cell phenotypes by FACS 7 days after the second co-immunization by the separation of $CD4^+$ T cells into $CD25^-$ or $CD25^+$ subtypes with multiple staining using anti-$CD4^+$-FITC, anti- $CD25^+$-PECy5 mAbs for the surfaces, then using anti-IL-10-APC and anti-Foxp3-PE mAbs for intracellular analyses. The results show that the population of $CD4^+CD25^-$ IL-10^+FoxP3^+ iTreg cells was increased in the mice co-immunization with pVAX-FSA1 + FSA1 compared with other groups (Fig. 1b). In contrast, as a positive control, $CD4^+CD25^+$ T cells from each group highly expressed both FoxP3 and IL-10, but there was no significant difference in their concentration. It suggests that FSA1 full-length cDNA used as the co-immunogen could also induce iTreg cells in mice effectively.

Immunosuppressive function of the induced iTreg cells was examined by the adoptive transferring and challenge analysis. Naïve C57BL/6 mice were adoptively transferred by 3×10^6 of $CD4^+CD25^-$ or 3×10^5 of $CD4^+CD25^+$ cells isolated from C57BL/6 mice co-immunized with the pVAX-FSA1 + FSA1, pVAX-FSA1 + BSA, Vector + FSA1 or without any vaccine, respectively. After 24 h, the mice were primed with flea antigen extracts formulated in alum once i.p. and their skin reaction examined 7 days later. As shown

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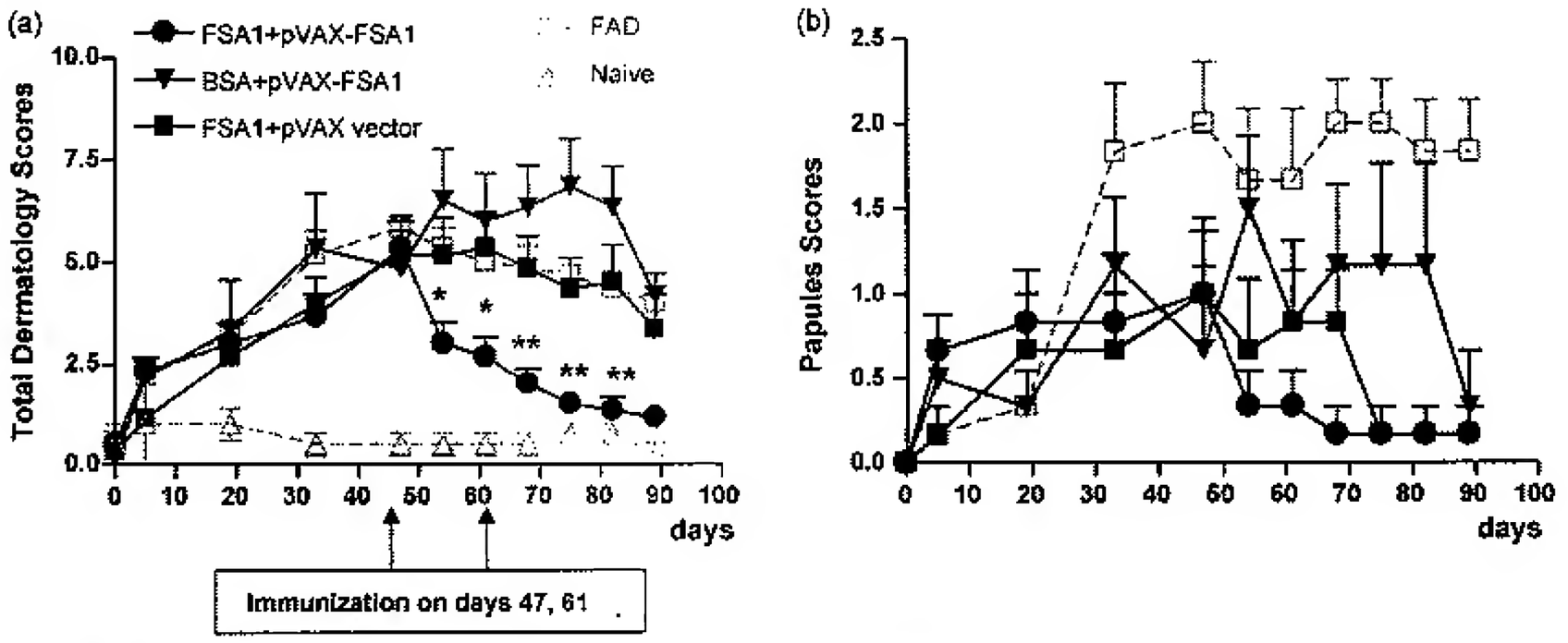

Fig. 3. Co-immunization of DNA and protein vaccines reduces the clinical symptom of FAD cats. (a) The total dermatology scores of FAD cats decreased after co-immunized with FSA1 + pVAX-FSA1. The scores of three notable lesions including the papules (b), crusts (c) and alopecia (d) are also decreased synchronously. $^{*}P<0.05$, $^{**}P<0.01$, compared with FAD model group. ($n=6$ cats per group).

in Fig. 1c, the $CD4^{+}CD25^{-}$ T cells from mice co-immunized with the pVAX-FSA1 + FSA1 and $CD4^{+}CD25^{+}$ T cells from all groups were able to suppress the development of AIH. In contrast, $CD4^{+}CD25^{-}$ T cells from other immunized groups did not suppress this AIH.

Together, both *in vitro* and *in vivo*, results are consistent and indicated that co-immunization with the DNA vaccine and its coding protein could induce the $CD4^{+}CD25^{-}$ iTreg cells with suppressive function against inflammatory T cells.

Fig. 4. Co-immunization inhibits the allergic responses including the IDT and number of mast cells at site of challenge in cats. (a) The FAD cats were intramuscularly co-immunized twice bi-weekly intervals with different regimens as indicated and subsequently challenged by the flea extracts, histamine as a positive control, or PBS as a negative control on days 7. The local reactions were measured at 20 min after the challenge, where the dashed lines were indicated as the average reaction from the histamine challenge. (b) Co-immunization suppressed T cell proliferative response to flea allergen in cats. Proliferation of T cells separated from peripheral blood mononuclear cells of cats were performed on days 7 after the second co-immunization. Proliferation assay was analyzed by MTT method.

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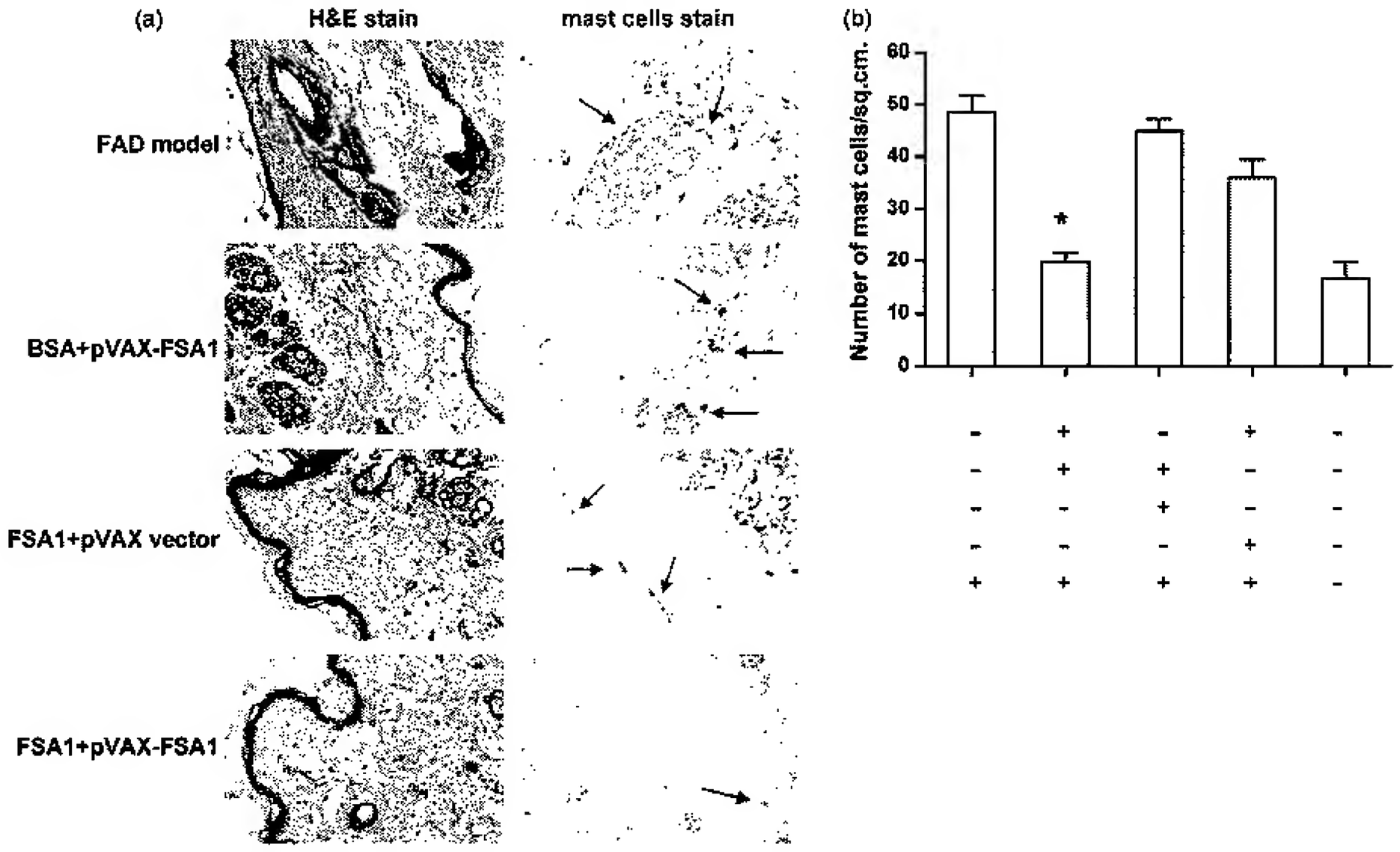

Fig. 5. Co-immunization inhibits the lymphocytes and mast cells infiltration. (a) Histology examination of skin biopsies from each group after the IDT at days 7 post the second immunization was performed with H&E and mast cell stainings. (b) The number of mast cells per cm^2 was also counted for 3 individuals independently in these groups. *$P < 0.05$, compared with FAD model group.

3.2. Therapeutic efficacy of co-immunization on clinical symptom in FAD cats

Having demonstrated that co-immunization induces iTreg cells, we next investigated if this protocol can be used in larger animals and might provide therapeutic benefit, clinically. In order to evaluate the therapeutic efficacy of this co-immunization approach against FAD in cats, these SPF cats were induced into FAD with seven rounds of flea infestations and scored by the dermatological assessments 4 days after each infestation. We observed that the clinical onset of disease was increased dramatically after the second infestation and reached to a plateau by the fourth cycle with the score around 6.0. The extra cycles of infestations did not contribute any higher responses (Fig. 2a). To evaluate the duration of FAD after the fourth infestation, a new group of SPF cats was used for the test. The result showed that the cats maintain their clinical onset status over 60 days following the 4th infestation (Fig. 2b) and retain allergic responses to the flea extract challenge (data not shown) over 90 days. This 60 days plateau duration would serve as the evaluation period for the co-immunization treatment.

To examine the efficacy of the co-immunization protocol of therapeutically treatment, cats were co-immunized twice on days 47 and 61 with pVAX-FSA1 + FSA1 as the test group, and co-immunized with pVAX-FSA1 + BSA or FSA1 + pVAX as the control groups after the 4 rounds of infestations. A certified veterinary doctor performed blinded assessments of clinical dermatology on days 0, 2, and then bi-weekly for the duration of the study, independently. As depicted in Fig. 3a, the dermatologic scores in the group co-immunized with FSA1 + pVAX-FSA1 were profoundly reduced from 6.0 to 1.5 ($P < 0.01$), compared with other groups immunized with the FSA1 + pVAX and pVAX-FSA1 + BSA, or with the untreated cats. These results demonstrate that the co-immunization protocol can ameliorate established FAD in cats. It also suggests that the other immunization combinations of DNA + protein using mismatched sequences do not induce iTreg cells in mice, and therefore could not suppress FAD clinically.

To correlate reduction of clinical disease by the co-immunization protocol, we analyzed each individual clinical symptom category, respectively. As depicted in Fig. 3b–d, the scores for papules and crusts are the most significantly reduced symptom in the co-immunized group compared with the other treated or untreated groups. Alopecia was also alleviated in the co-immunized group comparing to the other groups. However, the other clinical symptoms or lesions showed no significant difference after the co-immunization treatments (data not shown). Taking these observations together, we conclude that amelioration of FAD in cats by the twice-administered co-immunizations of pVAX-FSA1 + FSA1 is mainly due to the alleviation of papules, crusts and alopecia in the FAD cats.

3.3. Reduction of allergic reactions in FAD cats

Compared to other groups, cats immunized with the FSA1 + pVAX-FSA1 exhibited a significant reduction of these symptoms, which also correlated well with the significantly reduced level of skin reactions (Fig. 4a) and lower levels of T cell proliferative response (Fig. 4b). Since the level of infiltrated lymphocytes is a crucial factor contributing to local dermatitis [30–32], we collected the skin biopsies from the treated groups 20 min after the skin test on days 7, post second immunization. As depicted in Fig. 5a and b, the FSA1 + pVAX-FSA1 co-immunized cats showed fewer infiltrating lymphocytes and weaker mast cells activation compared with the other treated groups.

Since the allergen-specific IgE antibodies play a key role for allergic diseases [33–35], we also tested the change of FSA1 specific antibody level for the IgE (Fig. 6a) during the study. From the results of FAD model cats, we found the IgE level declined continually after ceasing the infestation. Comparing the IgE levels of each group, we

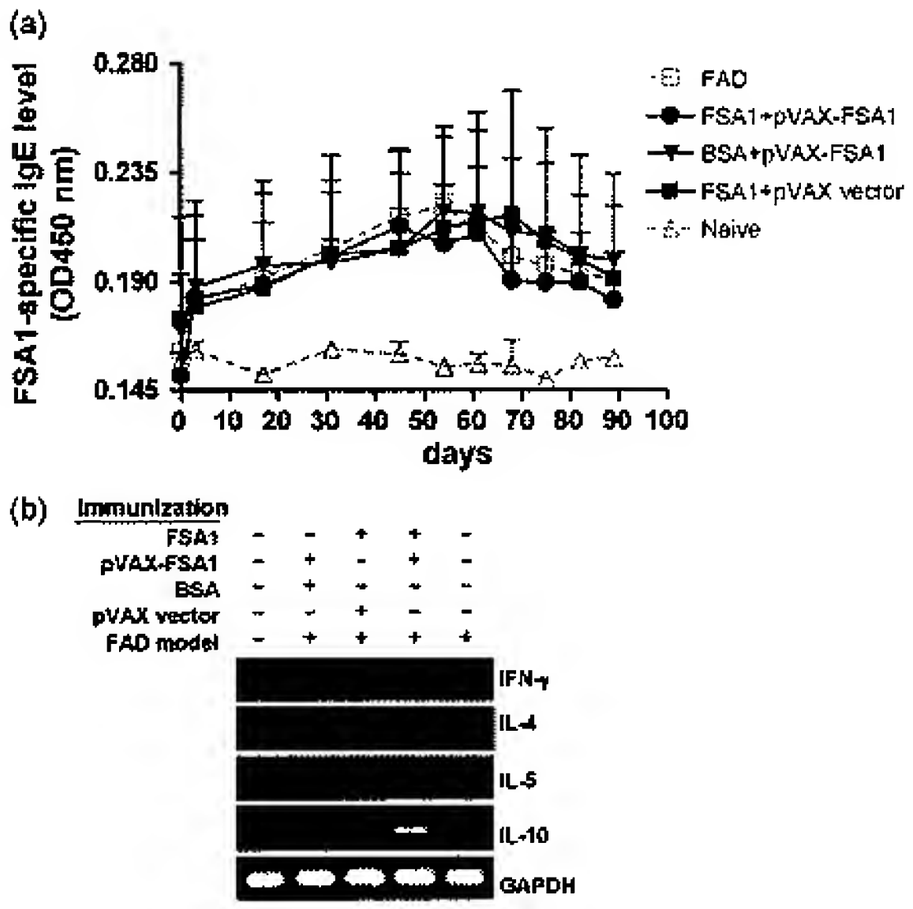

Fig. 6. Effects of co-immunization in cats on the levels of cytokines, IgE. Levels of IgE (a) specific to flea antigens were tested by ELISA on the 2 days after each infestation cycle. $^{*}P<0.05$, $^{**}P<0.01$ compared with the scores from FAD model group. ($n=6$ cats per group). (b) Cytokine production in T cells from cats PBMC on days 7 after the last co-immunization was examined by RT-PCR. The expression of GAPDH was served as an internal control of samples. Results are representative of at least three independent experiments.

observed no significant difference among all the groups, indicating that the co-immunization protocol may not directly contribute to eliminate existent IgE antibody. Nevertheless whether the co-immunization approach could in fact, inhibit the synthesis of IgE, is now under investigation.

Previous work has shown in other animals, that the IFN-γ associated with the TH1 response; IL-4 and IL-5 for the TH2 response, and IL-10 secreted by the Treg cells can be considered as immunosuppressive cytokines [36]. Because of the lack of anti-feline reagents for the detection of cytokines, we examined the cytokines expressions by RT-PCR assay using the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal control for RNA levels. As showed in Fig. 6b, the high level of expression for IL-5 increased in the FAD group and the mismatch groups, but not in the FSA1 + pVAX-FSA1 co-immunized group. As previously reported, Eotaxin-2 and IL-5 cooperate to regulate IL-13 production and eosinophilia [37], the lower level of IL-5, therefore, suggests a weaker allergic responses. Consistently with our previous study, we also observed that no differences at expression levels of IL-4 and IFN-γ were exhibited among all the groups (Fig. 6b), suggesting that the therapeutic effect might not be associated with the Th1 and Th2 responses in cats. iTreg cells may function via inhibiting the secretion of IL-5. On the other side, the elevated level of IL-10 expression was only observed in the FSA1 + pVAX-FSA1 co-immunized group significantly, suggesting an inhibitory response was elicited by this protocol.

4. Discussion

Our studies demonstrate that the co-immunization of FSA1 expressing DNA vaccine with its cognate-recombined protein together induces iTreg cells in mice, which are able to suppress cat FAD induced by flea infestations. These iTreg cells exhibit a phenotype of $CD4^{+}CD25^{-}FoxP3^{+}IL\text{-}10^{+}$ and suppress antigen-specific allergic reaction by reducing the allergen-specific T cells responses and allergic cytokine expressions.

As known, the previous reports had already found several types of regulatory T cells including Tr1, Th3 and nTreg cells. Each type of these Treg cells has respective hallmark phenotypes. For example, nTreg cells had $CD4^{+}CD25^{+}$ characteristic with expressing FoxP3 transcription factor constitutively [38,39]. On the other hand, Tr1 and Th3 could be induced from peripheral $CD4^{+}CD25^{-}$ T cell populations, but expression of FoxP3 is not necessary for them [40]. Recently some reports had found that $CD4^{+}CD25^{-}FoxP3^{+}$ Treg cells increased in patients with systemic lupus erythematosus or in aged animals [41]. However, the induction and expansion of Treg cells *in vivo*, particularly for those antigen-specific iTreg cells, has been proven to be difficult. Our protocol offers a method to induce or expand these specific Treg cells *in vivo*, and so that this would present a novel therapeutic strategy for addressing autoimmune and allergic diseases.

In this report, we induced an FAD model in cats, which was successful as the ones similarly shown in a previously reported FAD model in dogs. Compared to the two FAD models, we noticed some differences between them. First, the FAD cats showed that the papules, crusts and alopecia as the major lesions on the skin, instead of erythema, alopecia, excoriation in dogs. Secondly, there was no significant "flea triangle" on the cats abdomen (data not show) as there was in dogs. Finally, FAD cats showed significantly more eosinophilia in their peripheral blood, which was not observed in dogs [42]. These differences may be due to the dissimilar habits of these animals or to their intrinsic immune responses. This FAD model in cats induced by fleas provides for more stable symptoms after 4 cycles of infestation, and its duration lasts over 6 weeks, which makes a therapeutic vaccine easier to be evaluated in this animal model.

From a clinical vantage, our results showed that the co-immunization strategy could ameliorate many of the clinical symptoms and the inflammatory immune reactions of the FAD cats. This immunization strategy has the potential for a significant curative effect for treating major lesions of FAD in cats such as erythema, alopecia, and excoriation. Co-immunization with FSA1 + pVAX-FSA1 could also induce a lower IDT reaction and weaker T cells proliferation in cats. All of these inhibitions may be due to iTreg cells, which reduce secretion of the allergy-related cytokine IL-5, and increase the suppressive cytokines like IL-10. As reported previously, allergic reactions have been linked to low concentrations of serum IL-10 in patients with atopic dermatitis and food allergy [43]. In our previous work, the inhibition of iTreg cells can be blocked by the addition of anti-IL-10 antibody, suggesting that the IL-10 plays a key regulatory role in this novel adaptive Treg cell-mediated immuno-suppression. IL-4 is another Th2 key cytokine and is known to stimulate the B cells to secrete IgE antibody [44]. But from the RT-PCR result, the expression of IL-4 had not been reduced significantly. So we hypothesized that the inhibition mechanism of Treg cells may just suppress the IL-5 and IL-13 secretion.

In summary, we demonstrate that the co-immunization protocol with FSA1 DNA vaccine and its cognate-recombined protein induces antigen-specific T cell impairment via the induction of $CD4^{+}CD25^{-}FoxP3^{+}IL\text{-}10^{+}$ Treg cells in mice. Importantly, for the first time, we demonstrate that this protocol can also induce beneficial immunotherapeutic effect ameliorating FAD in cats in a clinical setting. The utility of this unique strategy against flea-induced dermatitis could lead to the important development of a potent vaccine in cats and dogs.

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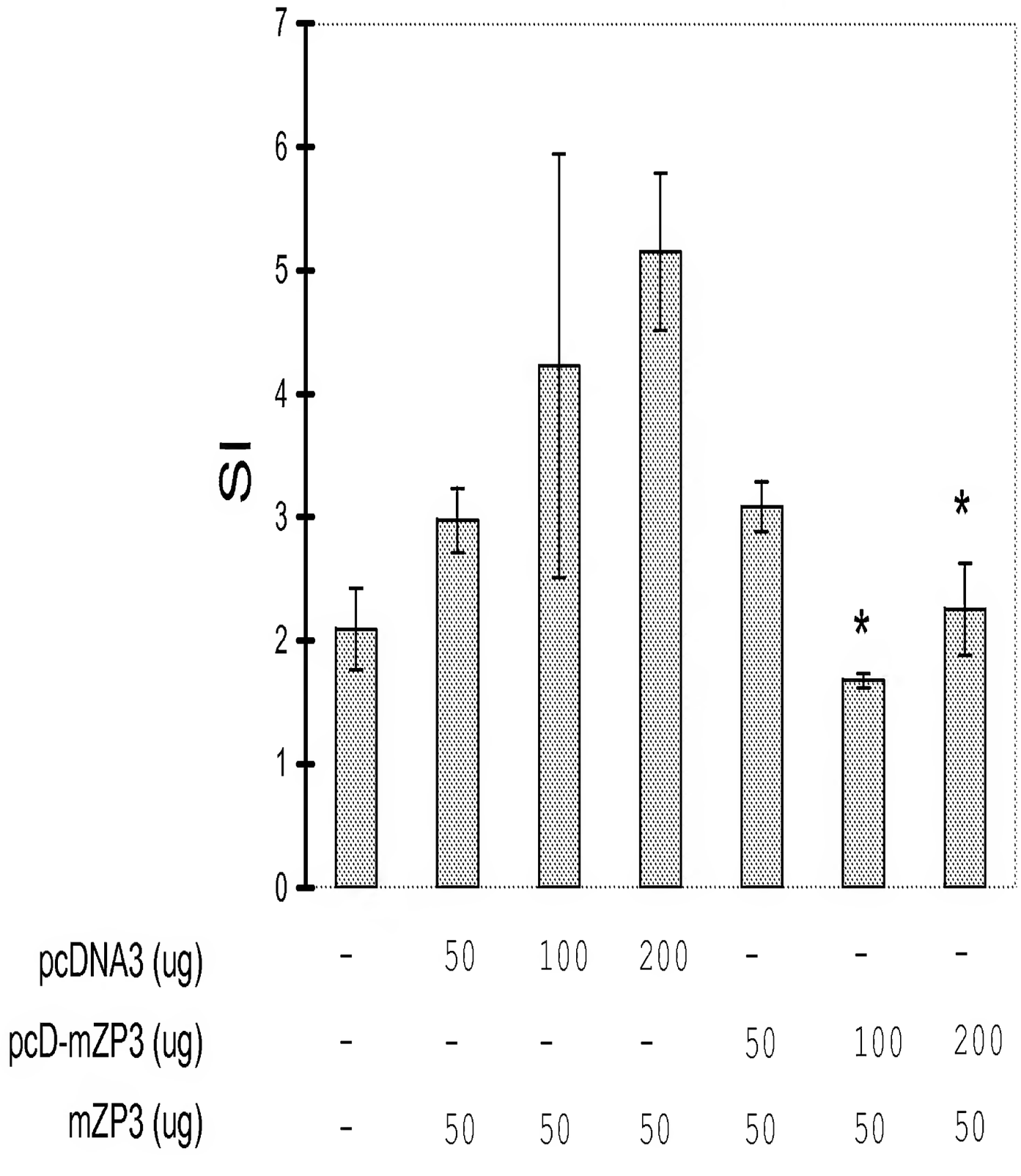
Exhibit 2: T cell proliferation after co-immunized different dose of pcD-mZP3 and mZP3

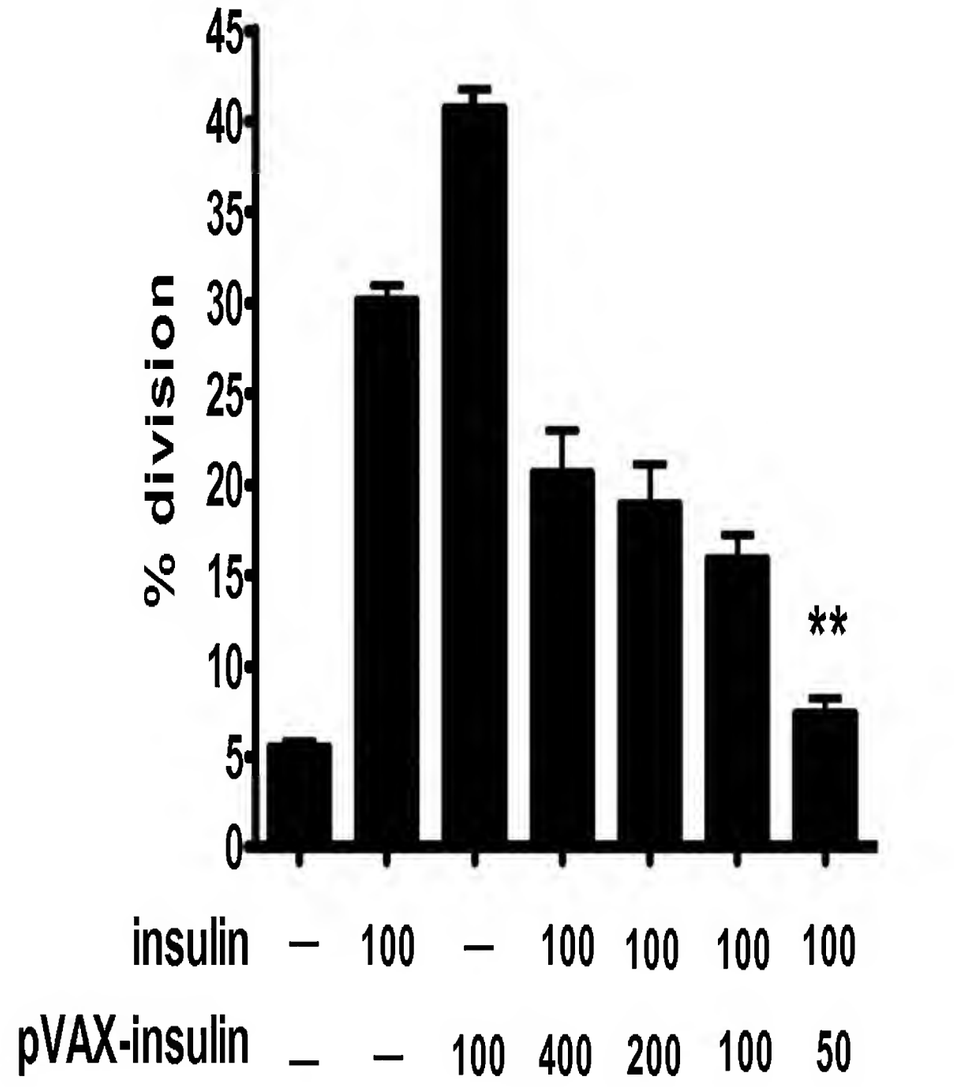


The C57BL/6 mice were immunized twice. T cell proliferation were done on day 3 after the second vaccination pcD-mZP3 +mZP3.

pcD-mZP3: encodes mouse zona pellucida 3 protein
mZP3: recombinant mouse zona pellucida 3 protein

Exhibit 3:

T cell proliferation activity determined by the division ability of T cells

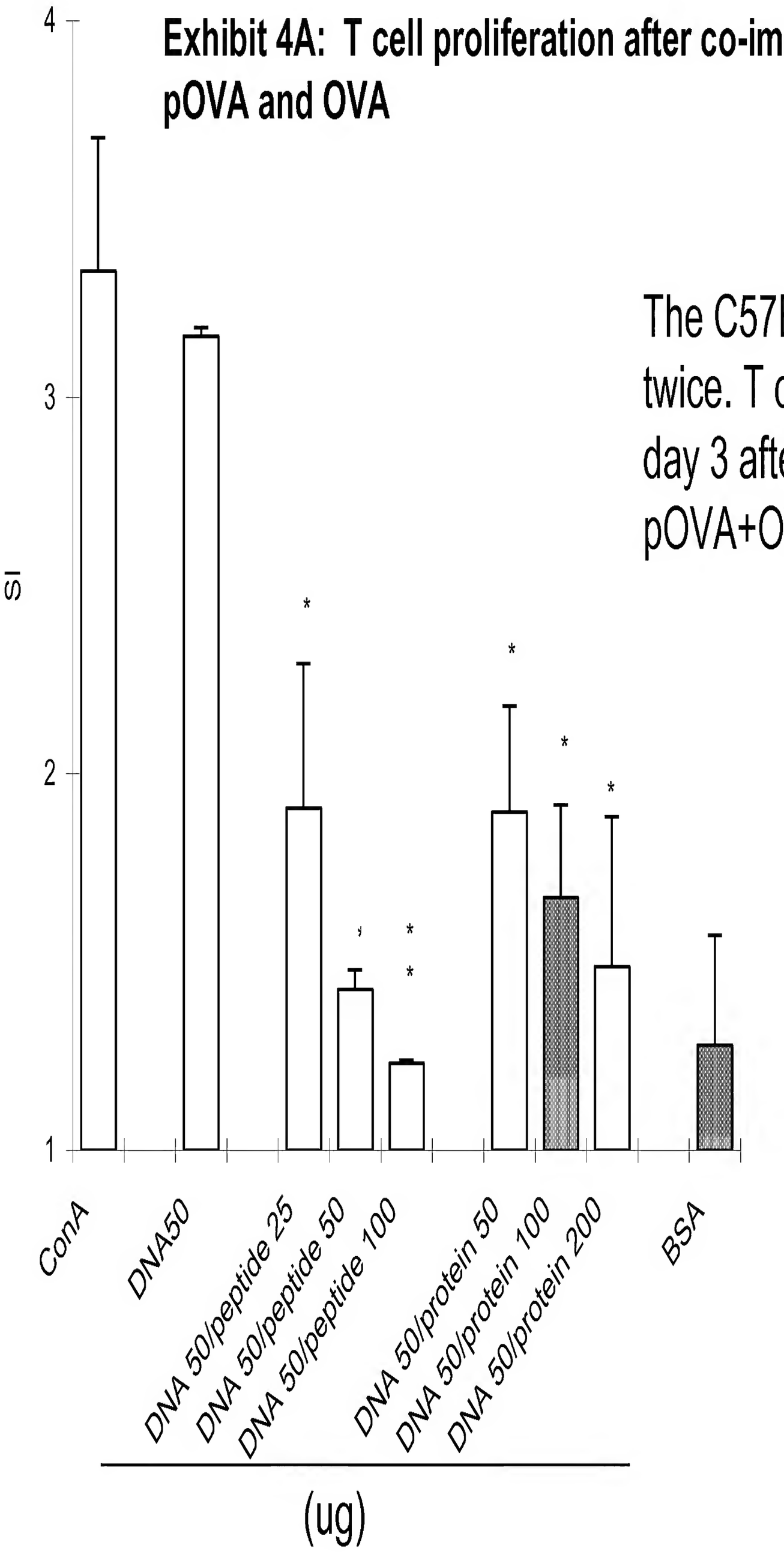


Protein: human recombinant insulin protein

DNA: pVAX-insulin encodes the human proinsulin

Method: different dose of ratio as followed to immunize BALB/c mice: 100 ug of protein insulin with 25, 50, 100, 200, 400 ug plasmid respectively or alone. Naïve mice is the negative antigens. 7 days after the second immunization T cell proliferation were detected.

Exhibit 4A: T cell proliferation after co-immunized different dose of pOVA and OVA

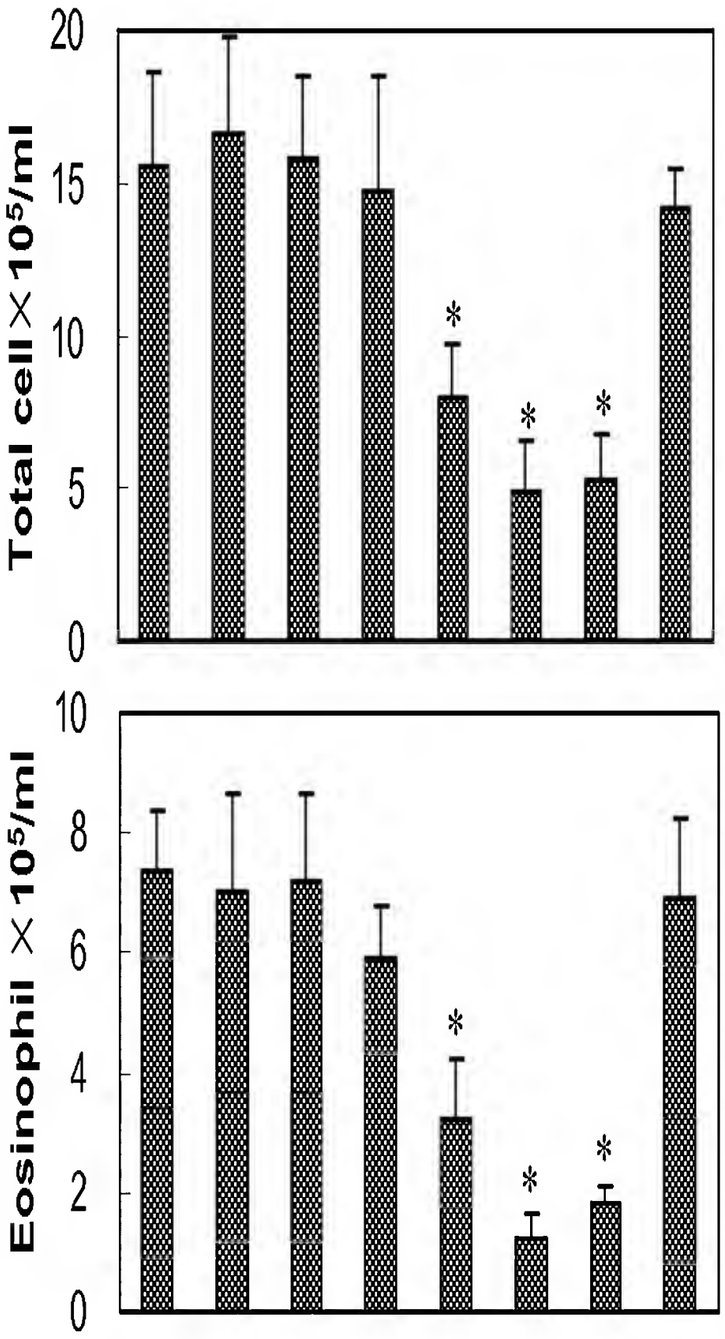


The C57BL/6 mice were immunized twice. T cell proliferation were done on day 3 after the second vaccination pOVA+OVA.

DNA: pOVA encodes OVA full-length
Peptide:OVA recombinant protein

Exhibit 4B:

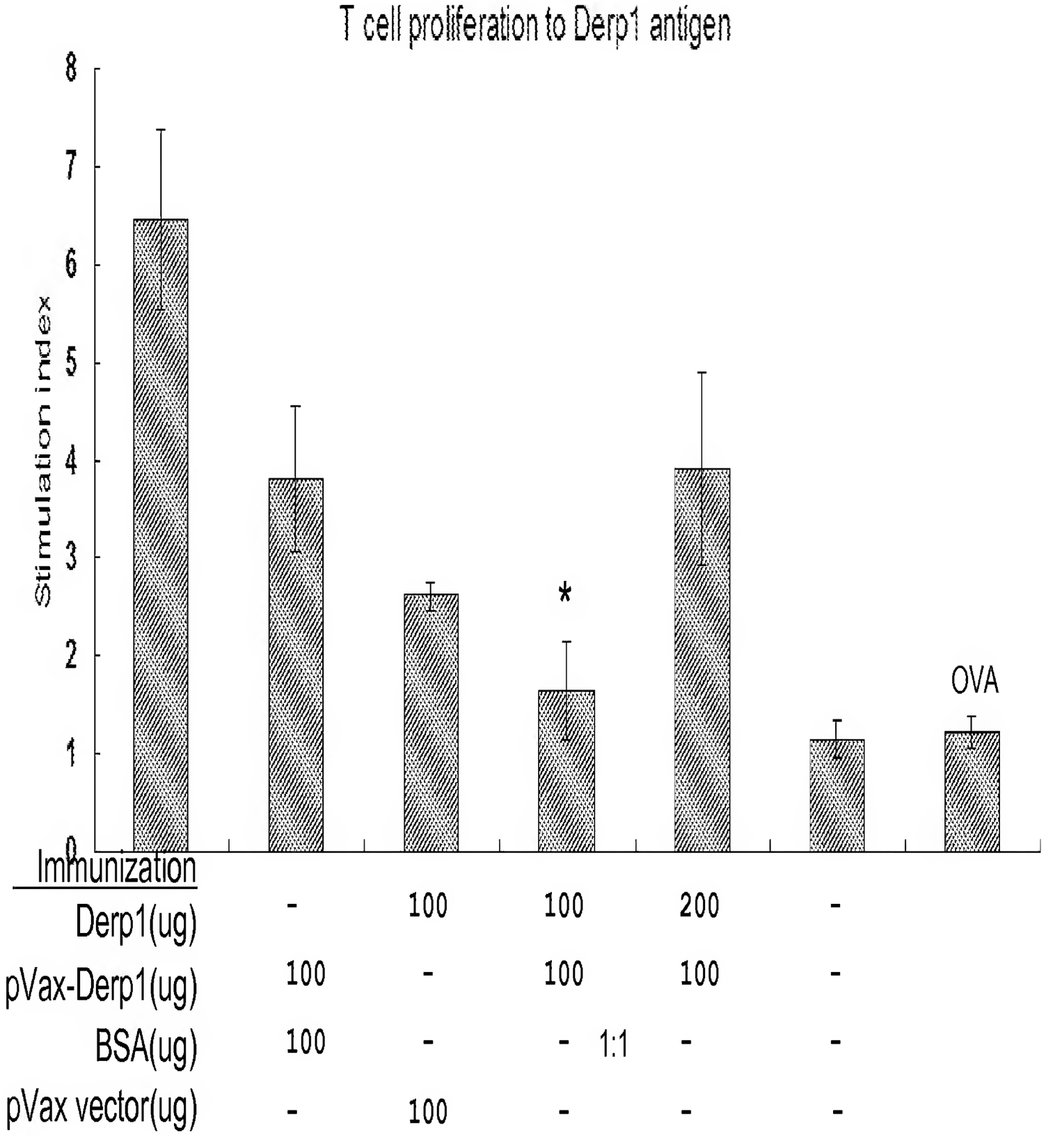
Asthma model



Pre-treatment								
pD323 (μg)	–	–	25	50	100	200	400	–
Pep323 (μg)	–	100	100	100	100	100	100	100
Vector (μg)	–	–	–	–	–	–	–	200
OVA sensitized	+	+	+	+	+	+	+	+
OVA challenged	+	+	+	+	+	+	+	+

Inhibition of the development of asthma by co-immunization of an OVA-peptide (pep323) and a DNA vaccine (pD323) encoding the same epitope. Mice were pre-treated with the indicated vaccination regimens intramuscularly on day -15 and -5, sensitized with OVA protein i.p. on day 0, and subsequently challenged with OVA intratracheally on days 8, 14, 16 and 18. The naïve mice without pre-treatment and sensitization, but OVA-challenged served as a negative control. Dose dependent suppression of infiltration of total cells and eosinophils into the BAL after co-immunization. *$p<0.05$ in comparison with the model group. *$p<0.05$ in comparison with the model group.

Exhibit 5: Der p 1 allergy model



The C57BL/6 mice were immunized twice. T cell proliferation were done on day 7 after the second vaccination pVAX-Derp1 and Derp 1

pVAX-Derp 1: DNA construct encodes Derp1 protein
Derp 1: recombinant Derp1 protein